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Journal of Chromatography B, 716 (1998) 251–255

JOURNAL OF CHROMATOGRAPHY B

Simultaneous measurement of blood and brain microdialysates of granisetron in rat by high-performance liquid chromatography with fluorescence detection

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Received 4 March 1998; received in revised form 25 May 1998; accepted 2 June 1998

Abstract

Simultaneous microdialysis probes in the blood and brain and sensitive high-performance liquid chromatography with fluorescence detection were used to examine the granisetron concentration in the jugular vein and frontal cortex of rats after drug administration. Two microdialysis probes were inserted into the right jugular vein and frontal cortex of male Sprague–Dawley rats to which granisetron (6 mg/kg, i.v.) had been administered. Dialysates were automatically collected using a microfraction collector. Samples were eluted with a mobile phase containing 25 m*M* acetate buffer (pH 4.8)–acetonitrile (72:28, v/v). Excitation and emission wavelengths were set at 305 and 360 nm, respectively, on a scanning fluorescence detector. The limit of quantification for granisetron was 0.5 ng/ml. The in vitro recovery of granisetron was 29.7±1.2% (n=6) for the jugular vein microdialysis probe and 6.1±0.5% (n=6) for the frontal cortex microdialysis probe. The increasing brain/blood concentration ratio of granisetron suggests that granisetron penetrates the blood–brain barrier.

Keywords: Granisetron

1. Introduction

Granisetron, endo-1-methyl-*N*-[9-methyl-9-azabicyclo(3.3.1)non-3-yl]-1*H*-indazole- 3-carboxamide, is a selective 5-HT_3 receptor antagonist which may have beneficial therapeutic effects in the treatment of vomiting and nausea resulting from cancer therapy [1–3]. For the determination of granisetron, a number of high-performance liquid chromatography techniques coupled with fluorescence detection [4–6] and mass spectrometric analysis [7] have been reported. However, most of these methods consume much time in sample clean-up procedures. Currently, microdialysis provides a clean sampling method for concentration measurements without the need for clean-up procedures [8,9].

Furthermore, there is another reason for choosing microdialysis: microdialysis is an in vivo sampling technique that allows the determination of drug concentration from protein unbound and extracellular

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0378-4347/98/$19.00
PII: S0378-4347(98)00274-6

space of most tissues [10–14]. This is important because the total drug concentration (protein-bound and unbound) in the blood does not reflect the concentrations at the cellular level, so that monitoring drug concentration in the interstitial space is crucial for understanding the time course of the anti-emetic activity of granisetron. In this study, we use microdialysis to provide near real-time analysis of granisetron in blood and brain dialysate samples after granisetron administration.

2. Experimental

2.1. Reagents

Granisetron hydrochloride was purchased from SmithKline Beecham Pharmaceuticals (Worthing, West Sussex, UK). The chromatographic reagent and gradient-grade solvents were obtained from Merck (Darmstadt, Germany). Triple de-ionized water (Millipore, Bedford, MA, USA) was used for all preparations.

2.2. Chromatography

The chromatographic system consisted of a chromatographic pump (BAS PM-80, West Lafayette, IN, USA), a Rheodyne Model 7125 injector equipped with a 20 μl sample loop and a fluorescence detector (Waters 474 scanning fluorescence detector, Milford, MA, USA). A sample was separated using a reversed-phase C_{18} column (150×4.6 mm; 5 μm; Cosmosil, Kyoto, Japan). Chromatography was performed at ambient temperature. The mobile phase consisted of 25 m*M* acetate buffer (pH 4.8)–acetonitrile (72:28, v/v). The 25 m*M* acetate buffer was prepared with 25 m*M* sodium acetate and the pH was adjusted to 4.8 by glacial acetic acid. The mixture was filtered with a 0.45 μm Millipore membrane. The optimal fluorescence response for granisetron was observed at excitation and emission wavelengths of 305 and 360 nm, respectively. Output data from the fluorescence were amplified and integrated on an integrator (SCI Chromatocorder 12, Tokyo, Japan).

2.3. Animals

Adult, male Sprague–Dawley rats (280–320 g) were obtained from the Laboratory Animal Center at National Yang-Ming University (Taipei, Taiwan). These animals were specifically pathogen-free and kept in environmentally controlled chambers (24±1°C and 12:12 h light–dark cycle). The rats were initially anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and anesthesia was continued throughout the experiment period.

2.4. Microdialysis experiments

Both blood and brain microdialysis systems used a CMA/100 microinjection pump (CMA, Stockholm, Sweden). For blood, CMA/20 microdialysis probes were used with a dialyzing membrane length of 10 mm and an outer diameter of 0.5 mm (CMA). The blood microdialysis probe was connected to the right jugular vein and then perfused with ACD solution (citric acid, 3.5 m*M*; sodium citrate, 7.5 m*M*; dextrose, 13.6 m*M*) at a flow-rate of 2 μl/min by a microinjection pump (CMA/100) [15].

After jugular vein cannulation, the rat was then mounted in a Kopf stereotaxic frame for brain microdialysis. Its body temperature was maintained at 37°C with a heating pad. The brain microdialysis probe (CMA/11) had a dialyzing membrane length of 3 mm and an outer diameter of 0.24 mm. The brain microdialysis probe perfused with Ringer's solution (147 m*M* Na^+; 2.2 m*M* Ca^{2+}; 4 m*M* K^+; pH 7.0) was implanted in the frontal cortex (coordinates: AP 2.2 mm; ML −3.2 mm; DV −3.0 mm) according to the Paxinos and Watson atlas [16]. The position of the probe was verified by a standard histological procedure at the end of the experiment [17].

Both blood and brain dialysis outflow samples were connected to a microfraction collector (CMA/140) and collected every 12 min. After dialysate levels had stabilized (approximately 2 h), the drug-free samples were collected and then granisetron (6 mg/kg) was administered intravenously via the femoral vein. Twenty microliters of each dialysate sample was assayed with the high-performance liquid chromatographic system. The microsyringe

and the 20 μl loop of the injector were washed with methanol before sample injection.

2.5. In vitro recovery

Both blood and brain microdialysis probes were calibrated by insertion in a tube containing 20 ng/ml granisetron and perfused with Ringer's solution. The perfusion media and pumping flow-rate were the same as in the above experiments. The probe recovery was calculated by dividing the concentrations in the dialysate (C_{out}) by the concentration in the tube (C_{in}) [18], that is: $\text{recovery}_{\text{in vitro}} = C_{out}/C_{in}$.

2.6. Method validation

All calibration curves were required to have a correlation value of at least 0.990. The intra-day and inter-day variabilities were determined by quantitating six replicates at concentrations of 0.5, 5, 10, and 20 ng/ml using the HPLC method described above on the same day and four different days, respectively. The accuracy was calculated from the nominal concentration (C_{nom}) and the mean value of observed concentrations (C_{obs}) as follows: accuracy (%) = $[(C_{nom} - C_{obs})/C_{nom}] \times 100$. The precision coefficient of variation (CV) was calculated from the observed concentrations as follows: precision (%) = [standard deviation (SD)/C_{obs}] × 100. The same data were used to determine both accuracy and precision.

The limit of detection (LOD) of the assay system was defined as the lowest concentration of the standard that could be measured with acceptable precision (CV < 20%). LOD was estimated using the external standard method [19] at a signal-to-noise ratio of 3:1. The limit of quantitation (LOQ) was in terms of the precision. LOQs of less than 15% were acceptable [20].

3. Results and discussion

3.1. Specificity of granisetron in blood and brain microdialysate

The method described in this paper provides an excellent separation from both blood and brain dialysates. The retention time of granisetron was found to be 5.7 min (Fig. 1). Fig. 1A shows a chromatogram of a blank blood dialysate. No interfering peaks were observed within the time frame in which granisetron was detected. Fig. 1B shows a chromatogram of a standard sample of granisetron (5 ng/ml). Fig. 1C shows a chromatogram of a blood dialysate sample containing granisetron (9.39 ng/ml) obtained from blood microdialysis 72 min after granisetron (6 mg/kg, i.v.) administration.

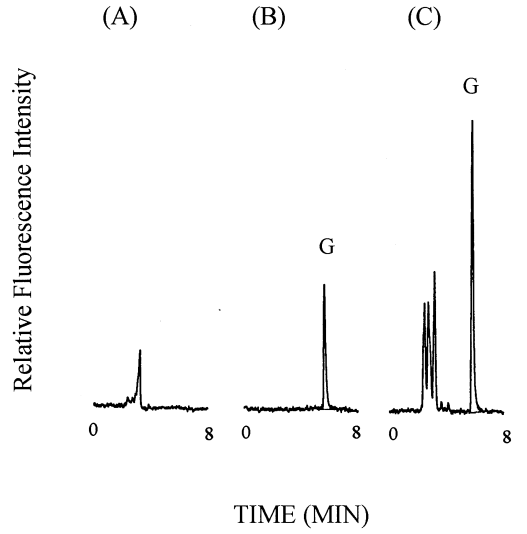

Fig. 1. Typical chromatograms of (A) a blank blood dialysate, (B) standard granisetron (5 ng/ml), and (C) a blood dialysate sample containing granisetron (9.39 ng/ml) collected from a rat blood microdialysis 72 min after granisetron (6 mg/kg, i.v.) administration. G, granisetron.

Fig. 2A shows a chromatogram of a blank brain dialysate. No discernible peaks were observed within the time frame in which granisetron was detected. Fig. 2B shows a chromatogram of standard granisetron (10 ng/ml). Fig. 2C shows a chromatogram of a brain dialysate sample containing granisetron (2.11 ng/ml) obtained from brain microdialysis 36 min after granisetron (6 mg/kg, i.v.) administration.

3.2. Limit of quantification, linearity and range

The peak detection limit of granisetron was 0.1 ng/ml at a signal-to-noise ratio of 3:1. The standard curve could be measured with acceptable accuracy

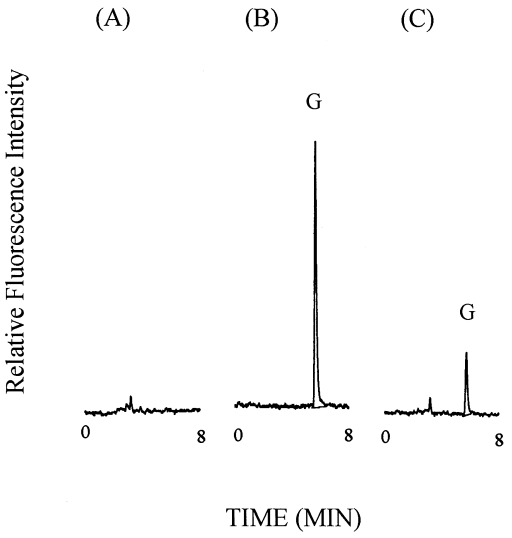

Fig. 2. Typical chromatograms of (A) a blank brain dialysate, (B) standard granisetron (10 ng/ml), and (C) a brain dialysate sample containing granisetron (2.11 ng/ml) collected from a rat brain microdialysis 36 min after granisetron (6 mg/kg, i.v.) administration. G, granisetron.

(LOQ<10%). The lowest observed LOQ of granisetron (9.35%) was found when $C_{nom}=0.5$ ng/ml in the inter-day assessment (Table 1). These results are in good agreement with those of Kudoh et al. [4] and Boppana [5], and demonstrate the high sensitivity of granisetron with the fluorescence detection method.

The calibration curve for granisetron was found to be linear throughout the range 0.5–100 ng/ml, which was the established range of interest. The linear regression equation was determined as $y=0.00035x-0.056$ ($n=12$, $r=0.99$), where y is the concentration (ng/ml) and x is the response in peak area.

Table 1
Intra-day and inter-day precision and accuracy of granisetron determination ($n=6$)

	Nominal concentration (C_{nom}, ng/ml)			
	0.5	5	10	20
Intra-day				
C_{obs}	0.52	5.06	9.99	20.11
SD	0.038	0.11	0.26	0.26
CV (%)[a]	7.31	2.17	2.58	1.28
Accuracy (%)[b]	−3.1	−1.17	+0.01	−0.54
Inter-day				
C_{obs}	0.52	5.03	10.02	20.12
SD	0.048	0.11	0.16	0.46
CV (%)[a]	9.35	2.09	1.55	2.29
Accuracy (%)[b]	−3.76	−0.53	−0.2	−0.63

[a]Precision CV (%)=[standard deviation (SD)/C_{obs}]×100.
[b]Accuracy (%)=[(C_{nom}−C_{obs})/C_{nom}]×100.

3.3. Precision and accuracy

The reproducibility of the method was determined by examining both intra-day and inter-day variabilities. The intra-day determination of granisetron at concentrations of 0.5, 5, 10, and 20 ng/ml were acceptable with CV (%) values of less than 10% (Table 1). The inter-day CV values for granisetron at the same concentrations were also less than 10% (Table 1).

3.4. Microdialysis

The in vitro recoveries of granisetron of the blood and brain microdialysis probes based on 20 ng/ml standard were 29.7±1.2% ($n=6$) and 6.1±0.5% ($n=6$), respectively. Since Telting-Diaz et al. [12] reported that the recovery is independent of both the concentration and the matrix (Ringer's solution, plasma or whole blood), based on the above report, subsequent probes were only calibrated using Ringer's solution. Furthermore, since the process depends on simple diffusion, the rate of transfer across the membrane is equivalent at all concentrations provided that other experimental conditions remain constant [21].

The dialysate samples collected over the first 60 min were discarded to allow recovery from the acute effects of the surgical procedure. The microdialysis sampling system and fluorescence liquid chromatographic system were then applied to the pharmacokinetic characterization and central nervous system distribution of granisetron in rats. Fig. 3 shows the simultaneously measured dialysate concentrations of granisetron in rats' blood and brain after granisetron (6 mg/kg, i.v.) administration. These data are uncorrected for the probe recovery rates. The average brain/blood ratio of granisetron concentration continued to increase from 12 to 144 min after drug administration. This enhanced ratio suggests that granisetron penetrated the blood–brain

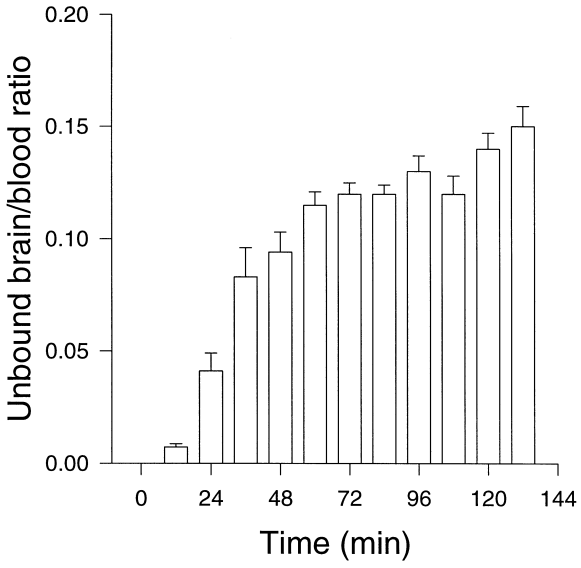

Fig. 3. Unbound brain/blood concentration ratio of granisetron after drug administration (6 mg/kg, i.v.) ($n=4$).

barrier. The current results are in good agreement with those of Bachy et al. [22], who found that [^{3}H]granisetron is capable of crossing the blood–brain barrier.

4. Conclusion

Our results show that it is feasible to measure simultaneously granisetron from blood and brain using two microdialysis probes. The microdialysis technique provides protein-free drug samples from blood and brain which can be injected directly into a chromatographic system for continuous in vivo monitoring. Further, compared with other in vivo methods for pharmacokinetic study, this sampling method causes less tissue damage in both the peripheral circulation system as well as the central nervous system.

Acknowledgements

This work was supported by a research grant (NSC87-2314-B-077-013) from the National Science Council, Taiwan.

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